

# A Comparison of Strategies To Stabilize Immunoglobulin F<sub>v</sub>-Fragments<sup>†</sup>

Rudi Glockshuber, Mark Malia, Ilse Pfitzinger, and Andreas Plückthun\*

Genzentrum der Universität München, Max-Planck-Institut für Biochemie, Am Klopferspitz, D-8033 Martinsried, FRG

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**ABSTRACT:** F<sub>v</sub>-Fragments of antibodies may dissociate at low protein concentrations and are too unstable for many applications at physiological temperatures. To stabilize F<sub>v</sub>-fragments against dissociation, we have tested and compared three different strategies on the F<sub>v</sub>-fragment of the well-characterized phosphocholine binding antibody McPC603 expressed and secreted in *Escherichia coli*: chemical cross-linking of the variable domains, introduction of an intermolecular disulfide bond, and construction of a peptide linker to produce a "single-chain" F<sub>v</sub>-fragment. All the linked fragments show hapten affinities nearly identical with that of the whole antibody independent of protein concentration and are significantly (up to 60-fold) stabilized against irreversible thermal denaturation. All genetically engineered linked F<sub>v</sub>-fragments can be obtained in native conformation in *E. coli*. The reported strategies for generating F<sub>v</sub>-fragments with improved physicochemical properties may extend their usefulness in biotechnology as well as in therapeutic and diagnostic applications.

Immunoglobulin F<sub>v</sub>-fragments (heterodimers consisting of only the variable domains V<sub>H</sub> and V<sub>L</sub>) are probably the minimal fragments of an antibody required for antigen binding activity. Their small size makes them interesting targets in the development of immunodiagnostic and immunotherapeutic applications since they may be expected to have better properties for penetration of solid tumor tissue as well as lower antigenicity and improved pharmacokinetics (Sedlacek et al., 1988). Furthermore, they are ideal models for protein engineering studies on antibodies as they provide the opportunity to investigate the binding properties of antibodies with a very small protein that is potentially amenable to structural analysis by NMR and crystallography. However, we have now found that F<sub>v</sub>-fragments show a surprisingly limited stability at low protein concentration and under physiological conditions. This fact prompted us to investigate ways of improving their stability.

The studies described here were carried out with the F<sub>v</sub>-fragment of the well-characterized phosphocholine binding IgA McPC603, for which the three-dimensional structure is known (Satow et al., 1986; Segal et al., 1974). We have previously developed a very convenient system for the expression of fully functional F<sub>v</sub>-fragments from *Escherichia coli* (Skerra & Plückthun, 1988; Plückthun et al., 1987) and have shown that the recombinant F<sub>v</sub>-fragment expressed in *E. coli* has nearly the same binding constant for the hapten as does the whole antibody (Skerra & Plückthun, 1988; see below).

Protein engineering on immunoglobulins may become an important part in the development of improved antibodies for diagnostics, therapy, and industrial applications such as protein purification or catalysis [reviewed, e.g., in Kraut (1988), Schultz (1988), Lerner and Bencovic (1988), and Plückthun et al. (1987)]. Rational approaches to the alteration of the binding properties of any protein are still at an early stage. Their development requires the availability of well-characterized model systems for antibody combining sites. With small, well-studied antigen binding fragments directly expressed in bacteria, random mutagenesis approaches may also be more easily carried out. All of these potential applications

require a full understanding of the stability properties of various recombinant antigen binding fragments. Furthermore, their antigen binding properties must be critically compared with the native antibody to evaluate the suitability of various recombinant fragments as model systems for the binding properties of the whole antibody. These questions are addressed for the F<sub>v</sub>-fragment of the antibody McPC603 in this paper.

## EXPERIMENTAL PROCEDURES

**Recombinant DNA Techniques and Protein Expression.** Recombinant DNA techniques were based on Maniatis et al. (1982). The antibody fragments were expressed in *E. coli* JM83 (Vieira & Messing, 1982) with a vector similar to that described (Skerra & Plückthun, 1988) but containing an M13 phage origin (Vieira & Messing, 1987; Skerra and Plückthun, unpublished results). Site-directed mutagenesis was carried out according to Kunkel et al. (1987).

**Protein Purification.** The recombinant antibody fragments were purified by phosphocholine affinity chromatography essentially as described previously (Skerra & Plückthun, 1988) except that the bacterial growth was performed at 20 °C and the cells were induced for 3 h before the harvest. The cells were then disrupted in a French pressure cell, and the soluble part of the lysate was directly applied onto the affinity column.

The F<sub>ab</sub>'-fragment of McPC603 was prepared essentially as described (Rudikoff et al., 1972).

**Cross-Linking.** The cross-linking with glutaraldehyde (0.10 M) was carried out in BBS buffer (0.16 M NaCl, 0.20 M borate/NaOH, pH 8.0) for 1 min at 20 °C. After addition of NaBH<sub>4</sub> to a final concentration of 0.10 M and incubation for 20 min (20 °C), the samples were dialyzed against BBS.

The glutaraldehyde-cross-linked F<sub>v</sub>-fragment was obtained on a preparative scale by cross-linking under the same conditions as in the analytical experiments at a protein concentration of 2 μM in the presence of 5 mM phosphocholine. The hapten was then removed by dialysis, and the cross-linked material was purified by affinity chromatography.

**Hapten Binding.** (a) *Fluorescence Measurements.* The fluorescence measurements were performed in BBS at 20 °C. The tyrosine and tryptophan fluorescence (excitation at 280 nm) was recorded for 5 s at 328 nm and averaged for each titration point. For the Scatchard analysis, the fluorescence

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\*To whom correspondence should be addressed.

change was recorded as a function of PC addition. The fraction of protein with bound hapten  $r$  was determined as  $(F - F_0)/(F_{\max} - F_0)$ , where  $F$  is the measured fluorescence,  $F_0$  the fluorescence without hapten, and  $F_{\max}$  the fluorescence in the presence of 10 mM PC. The concentration of free PC was calculated from the known protein concentration, the total PC concentration, and  $r$ . The protein molarity was determined by OD<sub>205</sub>, assuming (Scopes, 1982) an extinction coefficient of  $\epsilon_{0.1\%}^{1\text{cm}} = 31.0$ .

(b) *Equilibrium Dialysis.* The equilibrium dialysis measurements were carried out as described previously (Skerra & Plückthun, 1988).

*Protein Stability.* The  $F_v$ -fragments were incubated at identical protein concentrations (1.30  $\mu\text{M}$ ) in BBS at 37 °C. After different times of incubation (0–24 h), samples of 100  $\mu\text{L}$  were removed and centrifuged. Each supernatant (75  $\mu\text{L}$ ) was applied onto a 14% SDS-PAGE (Fling & Gregerson, 1986), and the amount of soluble protein was determined densitometrically with bovine serum albumin (BSA) as an internal standard (see Figure 1c). The first-order kinetics obtained had correlation coefficients of 0.99 and were used to obtain the half-lives in Table I.

*Other Methods.* The relative functional expression of the various fragments was determined as the approximate relative amounts purified by affinity chromatography from cells grown under identical conditions.

## RESULTS AND DISCUSSION

Preliminary results had suggested that the  $F_v$ -fragment of the antibody McPC603 might dissociate at high dilution (Plückthun et al., 1988, 1989). We have now examined this finding in detail by covalently cross-linking (Jaenicke & Rudolph, 1986) the variable domains of the  $F_v$ -fragment with glutaraldehyde at different protein concentrations. From the observed concentration-dependent dissociation equilibrium, we deduce that the equilibrium constant for the dissociation of the two domains of the  $F_v$ -fragment is on the order of  $10^{-6}$  M (Figure 1a).

To investigate the effect of the dissociation of the  $F_v$ -fragment on its apparent binding properties, we have analyzed its hapten affinity in more detail and compared it with that of the proteolytically prepared  $F_{ab}'$ -fragment of McPC603. This was achieved by fluorescence titration experiments, which are made possible by an increase in protein fluorescence induced by hapten binding (Glaudemans et al., 1977; Pollet & Edelhoch, 1973). The hapten-induced fluorescence change can only be observed when the excitation is carried out at 280 nm; at 295 nm there is essentially no observed fluorescence increase. This suggests the involvement of tyrosine residues in the process causing the fluorescence change.

The affinity constant of the  $F_{ab}'$ -fragment for phosphocholine of  $1.6 \times 10^5 \text{ M}^{-1}$  so obtained was found to be identical with the value reported for the whole antibody (Metzger et al., 1971). In contrast, the dissociation of the  $F_v$ -fragment led to distinctly lower apparent hapten binding constants of the  $F_v$ -fragment as measured by fluorescence, which were found to be dependent on protein concentration (Figure 2a). The curved appearance of the Scatchard plot is caused by the fact that the fluorescence measurement also records changes in domain association, which in turn depends on hapten binding. These results are entirely consistent with our previously reported affinity constant for the  $F_v$ -fragment (Skerra & Plückthun, 1988) of  $1.2 \times 10^5 \text{ M}^{-1}$ , which had been determined by equilibrium dialysis at high protein concentration (8.5  $\mu\text{M}$ ). This measured value is reproduced by calculation, assuming an intrinsic hapten binding constant identical with that of the

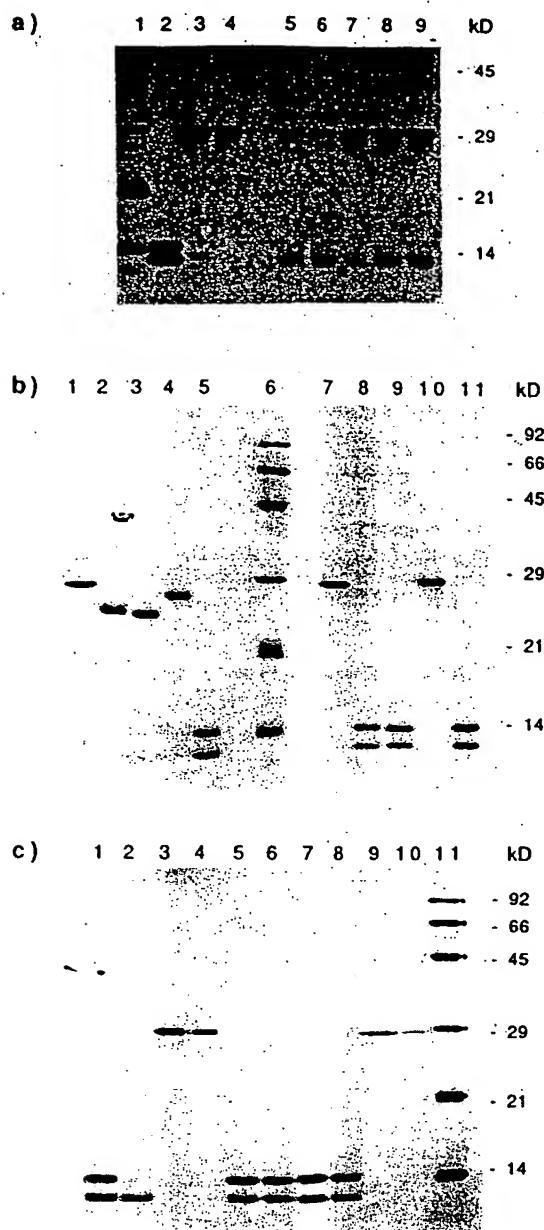


FIGURE 1: (a) Covalent cross-linking of the native  $F_v$ -fragment with glutaraldehyde at different protein concentrations. A silver-stained SDS-PAGE (14%) is shown: (lane 1) protein molecular size marker; (lane 2) native  $F_v$ -fragment; (lanes 3 and 4)  $F_v$ -fragment, cross-linked in the presence of 5 mM phosphocholine at protein concentrations of 0.8 and 12.0  $\mu\text{M}$ , respectively; (lanes 5–9)  $F_v$ -fragment, cross-linked at protein concentrations of 0.8, 1.5, 3.0, 6.0, and 12.0  $\mu\text{M}$ , respectively, but in the absence of the hapten. The cross-linking with glutaraldehyde was carried out as described under Experimental Procedures. (b) Purification of the native  $F_v$ -fragment and of the various covalently linked  $F_v$ -fragments. An SDS-PAGE (14%) stained with Coomassie brilliant blue is shown. The samples in lanes 6–11 were reduced by boiling with  $\beta$ -mercaptoethanol, and the samples in lanes 1–5 were not reduced. (Lanes 1 and 7) Single-chain  $F_v$ -fragment; (lanes 2 and 8) disulfide-linked mutant 56–106; (lanes 3 and 9) disulfide-linked mutant 55–108; (lanes 4 and 10) chemically cross-linked  $F_v$ -fragment; (lanes 5 and 11) native  $F_v$ -fragment; (lane 6) protein molecular size marker. (c) Thermal stability of the native and the cross-linked  $F_v$ -fragments. An SDS-PAGE (14%) stained with Coomassie brilliant blue is shown. (Lanes 1 and 2) Native  $F_v$ -fragment; (lanes 3 and 4) chemically cross-linked  $F_v$ -fragment; (lanes 5 and 6) mutant 55–108; (lanes 7 and 8) mutant 56–108; (lanes 9 and 10) single-chain  $F_v$ -fragment; (lane 11) protein molecular size marker. Identical amounts of the soluble fraction of the protein solutions before (corresponding left lanes) and after (right lanes) a 24-h incubation at 37 °C were applied to the gel. The internal standards were omitted for the gel shown here as an example of a 24-h incubation.

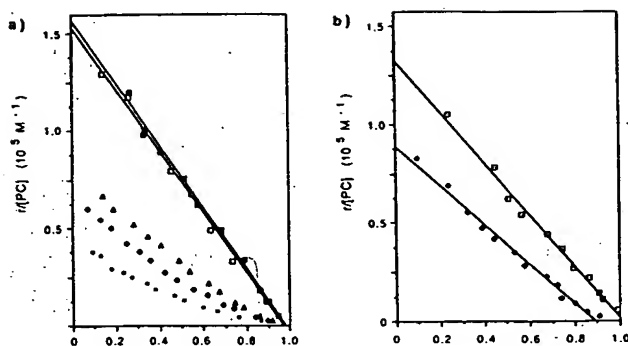


FIGURE 2: Fluorescence changes upon binding phosphocholine (PC). (a) Scatchard plot of the binding of PC to the native  $F_v$ -fragment at various protein concentrations [ $\Delta$ ] 2.89, [ $\diamond$ ] 0.96, and (small solid squares) 0.32  $\mu M$ ] compared with the proteolytically prepared  $F_{ab}'$ -fragment [large solid squares] 0.96  $\mu M$ ] and the chemically crosslinked  $F_v$ -fragment [open squares] 0.96  $\mu M$ ]. (b) Scatchard plot of PC binding to the genetically engineered cross-linked  $F_v$ -fragments: (□) single-chain  $F_v$ -fragment; (◆) 55-108. The fluorescence measurements were performed as described under Experimental Procedures.  $r$  denotes the fraction of antibody fragment with bound hapten, and  $[PC]$  denotes the concentration of free phosphocholine.

Table I: Properties of Covalently Linked  $F_v$ -Fragments

fragment <sup>a</sup>	$K_{assoc}^{PC}$ ( $10^3 M^{-1}$ )		relative functional expression in vivo	half-life of denaturation at 37 °C (h)
	fluor	eq dial		
native $F_v$ (wt)	b	1.2 <sup>c</sup>	1.0	1.3 <sup>d</sup>
cl $F_v$	1.6	nd		25
55-108	1.0	1.0	0.2	88
56-106	nd <sup>e</sup>	0.7	0.2	70
sc $F_v$	1.3	nd	0.5	15

<sup>a</sup>The fragments are the unmodified  $F_v$ -fragment (denoted native  $F_v$ ), the glutaraldehyde-cross-linked  $F_v$  (cl  $F_v$ ), the disulfide-linked fragments L55-H108 (55-108) and L56-H106 (56-106), and the single-chain  $F_v$ -fragment (sc  $F_v$ ). <sup>b</sup>Dependent on protein concentration because of the dissociation at low protein concentration (see text). <sup>c</sup>Measured at 8.5  $\mu M$  (Skerra & Plückthun, 1988). <sup>d</sup>Denaturation of  $V_H$ . <sup>e</sup>No fluorescence change; see text.

$F_{ab}'$ -fragment and the measured  $V_H:V_L$  dissociation constant for the  $F_v$ -fragment of  $1 \times 10^{-6} M$ .

We also observed a large difference between the thermal stability of the  $F_v$ -fragment and the  $F_{ab}'$ -fragment. While the  $F_{ab}'$ -fragment is fully stable at 37 °C for extended periods (data not shown), the  $V_H$  chain of the  $F_v$ -fragment rapidly and irreversibly denatures and precipitates (Figure 1c and Table I). This low stability makes the  $F_v$ -fragment unsuitable for many technical or medical applications. While the denaturation of  $V_H$  is rapid, the denaturation of the  $V_L$  chain is much slower. The different stabilities of the variable domains and the different stabilities of the  $F_v$ - and  $F_{ab}'$ -fragments are consistent with a pathway of protein denaturation in which the  $F_v$ -fragment dissociates first and then the domains unfold. These considerations led us to the hypothesis that the thermal stability of the  $F_v$ -fragment might be increased by a stronger association between  $V_L$  and  $V_H$ .

We therefore utilized three different strategies to stabilize the well-characterized  $F_v$ -fragment of the antibody McPC603 against dissociation by covalently linking the variable domains. Furthermore, in a covalently cross-linked fragment the hapten binding behavior should simply reflect the intrinsic binding properties of the whole antibody.

In the first approach, the  $F_v$ -fragment of McPC603 purified from *E. coli* was cross-linked with glutaraldehyde (Jaenicke & Rudolph, 1986) in the presence of phosphocholine. The cross-linked material was obtained with an overall yield of

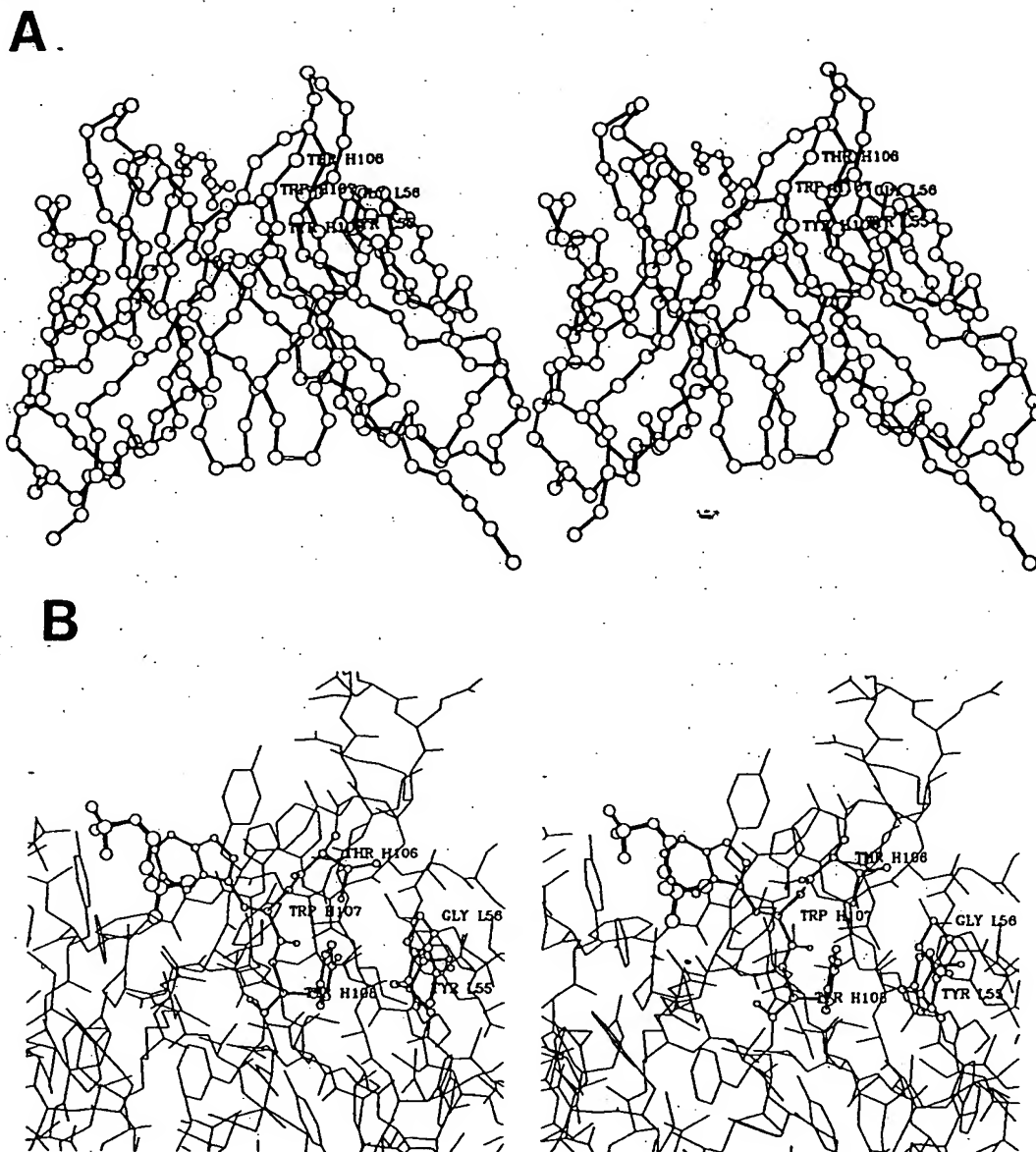
about 80% after purification to homogeneity (Figure 1b) with a phosphocholine affinity column (Chesebro & Metzger, 1972).

In a second approach, we constructed two different  $F_v$ -fragments each containing an intermolecular disulfide bond. To locate appropriate positions for cysteine residues, a collection of disulfide bonds from the protein data base was evaluated (Pabo & Suchanek, 1986). All possible amino acid pairs at the  $V_L:V_H$  interface whose main-chain atoms had RMS deviations of less than 2.0 Å from any member of this collection were identified. Of these candidate pairs, residues close to the hapten binding site and those involving proline residues were not considered. We report here the results on two molecules that were obtained by site-directed mutagenesis (Kunkel et al., 1987) [55-108, L55 Tyr → Cys, H108 Tyr → Cys; 56-106, L56 Gly → Cys, H106 Thr → Cys (Figure 3)]. We found that the intermolecular disulfide bond in both molecules had formed in the periplasm *in vivo*. In both mutant proteins, all the material isolated from a phosphocholine affinity column was covalently linked (Figure 1b), and all the covalently linked soluble material, as detected by Western blot from a nonreducing gel, binds to the affinity column (data not shown).

In the third approach, we constructed a secreted single-chain  $F_v$ -fragment. The intragenic region between  $V_H$  and  $V_L$  of the artificial operon (Skerra & Plückthun, 1988) as well as the signal sequence of the downstream gene encoding  $V_L$  was replaced by a DNA fragment encoding the sequence (Gly-Gly-Gly-Ser)<sub>3</sub> by site-directed mutagenesis. This  $F_v$ -fragment is thus encoded by a single peptide segment and contains one signal sequence. This signal is the same as that used for  $V_H$  in all other  $F_v$ -fragments reported here. This single-chain  $F_v$ -fragment was found to be secreted normally and could also be directly purified by affinity chromatography in a single step (Figure 1b). In contrast to the previously reported expression strategy of similar molecules (Bird et al., 1988; Huston et al., 1988), no *in vitro* refolding is necessary in our expression system.

The amounts of the various functional  $F_v$ -fragments obtained from *E. coli* were compared. None of the described strategies to improve the association of  $V_L$  and  $V_H$  resulted in higher yields of native cross-linked  $F_v$ -fragments than that obtained for the wild-type fragment with two unlinked chains (Table I). From this finding we deduce that it is *not* the association of the variable domains that limits the achievable expression level of functional  $F_v$ -fragments in *E. coli*. Rather, this result is consistent with an alternate view, that the amount of correctly folded  $F_v$ -fragment is determined by the folding process of the single domains both *in vivo* and *in vitro* (Hochman et al., 1976). The single-chain strategy therefore offers no particular advantage as far as the expression yield in *E. coli* is concerned. The single-chain fragment has advantages in stability (see below), however, and the secretion system described here provides an easier access to such linked fragments.

We then determined the hapten binding constants of all cross-linked  $F_v$ -fragments by fluorescence titration (Figure 2 and Table I). The affinities for phosphocholine were found to be essentially the same for the  $F_{ab}'$ -fragment and the chemically cross-linked  $F_v$ -fragment, which demonstrates that the intrinsic binding property of McPC603 is fully retained in its  $F_v$ -fragment. The hapten affinities of all other cross-linked species were only slightly reduced. Furthermore, the affinity constants of all cross-linked  $F_v$ -fragments were independent of protein concentration in contrast to those of the native  $F_v$ -fragment.



**FIGURE 3:** Position of the engineered intermolecular disulfide bonds. The pairs of residues Tyr L55 and Tyr H108 or Gly L56 and Thr H106 were changed to cysteines. The hapten phosphocholine is shown as a ball-and-stick model in both panels. In (A), only the  $\alpha$ -carbon backbone is shown with the  $V_L$  domain having open bonds and  $V_H$  having filled bonds. The residue number is given on the right of the corresponding  $\alpha$ -carbon atom. In (B), a close-up view of the region around the mutated residues is shown. The residues Tyr L55, Tyr H108, Gly L56, and Thr H106 (which were changed to cysteines) as well as Trp H107 are highlighted as ball-and-stick objects. The residue number is given on the right of the corresponding  $\alpha$ -carbon atom. The coordinates are those of Davies and co-workers (Satow et al., 1986; Segal et al., 1974), and the plot program of Lesk and Hardman (1982) was used.

Surprisingly, no fluorescence change at all was observed for the mutant protein 56–106 upon addition of phosphocholine, although this protein bound to the phosphocholine affinity column and therefore could be purified to homogeneity. In contrast, mutant 55–108, in which two tyrosine residues were changed to cysteines, showed normal fluorescence changes. To clarify the behavior of these mutant proteins, their binding affinity to phosphocholine was determined by equilibrium dialysis. The value obtained for 56–106 ( $0.7 \times 10^5 \text{ M}^{-1}$ ; Figure 4, Table I) shows that this mutant protein is able to bind phosphocholine with only a 2-fold reduction in hapten affinity compared with the native antibody. Both methods (fluorescence and equilibrium dialysis) gave identical values for the other disulfide mutant 55–108, validating both approaches. This protein was also found to bind the hapten with a similar affinity as the whole antibody. The reason for the unexpected lack of hapten-induced fluorescence change of the mutant protein 56–106 may be a tethering of the CDR-3 loop of  $V_H$

by the disulfide bond involving H106 (Figure 3). Tryptophan H107, which is directly in contact with the hapten, or another aromatic residue in the neighborhood, may be restricted in its movement by a disulfide bond at the tip of the loop (H106) but less so by a disulfide bond further away from the tip (H108) in the mutant 55–108.

Upon addition of the hapten to saturation, the change in tyrosine fluorescence of both the  $F_{ab}'$ -fragment and the chemically cross-linked  $F_v$ -fragment is a very fast process whose kinetics cannot be resolved without recourse to rapid-mixing techniques. In contrast, the natural  $F_v$ -fragment, the single-chain  $F_v$ -fragment, and the disulfide-linked  $F_v$ -fragment give rise to a fast step followed by a slow fluorescence change apparently displaying first-order kinetics on a time scale of minutes, similar to previously reported results (Watt & Voss, 1979). The rate of this slow process does not appear to depend on protein concentration. The physical basis of this slow fluorescence change needs to be further investigated. It might

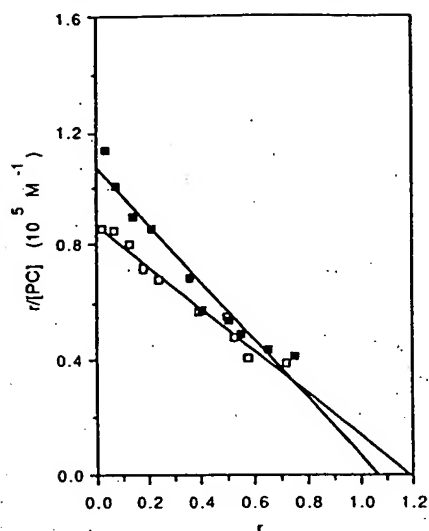


FIGURE 4: Scatchard plot of the equilibrium dialysis data for the binding of phospho[ methyl-<sup>14</sup>C]choline to the disulfide mutant proteins 55-108 and 56-106.  $r$  denotes the fraction of antibody fragment with bound hapten, and  $[PC]$  denotes the concentration of free phosphocholine. (■) Mutant protein 55-108 at 10  $\mu$ M; (□) mutant protein 56-106 at 10  $\mu$ M.

be reflected in the slight differences in apparent hapten binding constants and may also involve movement of the CDR3 loop in  $V_H$ .

The thermal stability of the various covalently linked  $F_v$ -fragments was measured by recording the decrease of soluble protein on incubation at 37 °C (Figure 1c). We found that all cross-linking strategies led to a strong stabilization of the corresponding proteins against irreversible denaturation. The data were analyzed according to first-order kinetics (Table I). The single-chain  $F_v$ -fragment, the least stable cross-linked protein, is still 10-fold more stable than the natural  $F_v$ -fragment. The disulfide-linked mutants 55-108 and 56-106 were found to be the most stable species with a 60- and 50-fold increase in the half-life at 37 °C, respectively. While  $V_H$  is much less stable than  $V_L$  in the natural  $F_v$ -fragment, the covalent linking improves the thermal stability of the linked fragments to at least that of  $V_L$  (Figure 1c).

We conclude from these results that the stability of the  $F_v$ -fragment of McPC603 is influenced by the interaction of the variable domains  $V_L$  and  $V_H$ . The association constants of antibody  $F_v$ -fragments that have been reported (Hochmann et al., 1976; Klein et al., 1979; Home et al., 1982) range from  $10^8$  to  $10^5$  M<sup>-1</sup>, and there is probably no strong natural selection for the interactions of the variable domains of antibodies due to the simultaneous association of the constant regions of both chains. Since hapten binding and domain association are mutually dependent, the analysis of hapten binding at low concentrations of  $F_v$ -fragment is rather complex. We show here that, for detailed hapten binding studies involving site-directed mutagenesis, the chemically cross-linked  $F_v$ -fragment is especially suitable as its binding properties are essentially identical with those of the  $F_{ab}'$ -fragment, straightforward to analyze, and probably directly reflect the intrinsic hapten affinity constants. On the other hand, all three reported strategies for preventing the dissociation of  $V_L$  and  $V_H$  lead to  $F_v$  molecules with dramatically improved thermal stabilities with the disulfide-linked mutants giving the most pronounced improvement. The greatly increased lifetime at physiological temperatures of all covalently linked  $F_v$ -fragments could make such fragments very promising agents for medical and biotechnological applications.

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## Organization and Expression of the Rat D<sub>2A</sub> Receptor Gene: Identification of Alternative Transcripts and a Variant Donor Splice Site<sup>†</sup>

Karen L. O'Malley,<sup>\*,†</sup> Kenneth J. Mack,<sup>†</sup> Kuan-Yun Gandelman,<sup>†</sup> and Richard D. Todd<sup>§</sup>

Department of Anatomy and Neurobiology and Departments of Psychiatry and Genetics, Washington University School of Medicine, 660 South Euclid Avenue, St. Louis, Missouri 63110

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**ABSTRACT:** We have recently reported the creation of a cell line expressing D<sub>2</sub> receptors encoded by a gene distinct from that described by Bunzow et al. [Bunzow, J. R., Van Tol, H. H. M., Grandy, D. K., Albert, P., Salon, J., Christie, M., Machida, C. A., Neve, K., & Civelli, O. (1988) *Nature* 336, 783-787]. To provide a framework for understanding structural differences between these and other G-protein-coupled receptors, the structure of the rat gene coding for the Bunzow et al. cDNA (called D<sub>2A</sub> here) was delineated. The D<sub>2A</sub> gene contains eight exons and spans at least 50 kb. Sets of oligonucleotide primers were used in combination with the polymerase chain reaction (PCR) to determine the presence of alternative transcripts within the introns. In contrast to other G-protein-coupled receptors, the D<sub>2A</sub> gene undergoes alternative RNA processing within intron 5, resulting in an insertion of 29 amino acids to the predicted 415 amino acid sequence of the D<sub>2A</sub> protein. By use of the PCR assay the relative abundance and tissue distribution of the alternative D<sub>2A</sub> transcripts (herein termed D<sub>2A415</sub> and D<sub>2A444</sub>) were determined. A variant donor splice site was also identified at the end of exon 4, a GC dinucleotide instead of the canonical GT. The variant dinucleotide was also present in the mouse but not in the human D<sub>2A</sub> gene.

**D**opamine receptors have been widely studied due to their proposed roles in the treatment and etiology of many neuropsychiatric disorders. Pharmacological and physiological studies have defined two principle types of dopamine receptors, D<sub>1</sub> and D<sub>2</sub>, each with distinct pharmacological binding profiles, signal transduction systems, and sites of localization (Hamblin et al., 1984; Seeman et al., 1985; Kebabian, 1986; Stoof & Kebabian, 1984; Freedman & Weight, 1988; Enjalbert et al., 1988). Bunzow et al. (1988) have reported the cloning of a rat cDNA with the expression characteristics of a D<sub>2</sub> receptor. This clone is a member of the G-protein<sup>1</sup>-coupled receptor family.

Recently we described a strategy for cloning cell surface proteins for which only radioligands are available (Todd et al., 1989). Using this technique, we isolated a cell line expressing a membrane-bound protein with the pharmacological characteristics of a D<sub>2</sub> receptor. With polymerase chain reaction (PCR) analysis we have shown that the expressed D<sub>2</sub> receptor is not the product of the Bunzow et al. D<sub>2</sub> receptor gene (Todd et al., 1989). Therefore, there must be at least two genes that produce D<sub>2</sub> receptor subtypes designated here as D<sub>2A</sub> (Bunzow et al., 1988) and D<sub>2B</sub> (Todd et al., 1989).

In order to characterize the functional and evolutionary relationships between these receptors and as the first step in developing experimental systems for studying the regulation

of D<sub>2</sub> receptor gene expression during development and differentiation, we have isolated and characterized the rat D<sub>2A</sub> gene.

### EXPERIMENTAL PROCEDURES

**Materials.** Most enzymes were purchased from Promega Biotech. Sequanase and AmpliTaq were from U.S. Biochemicals. Nylon membranes were from Schleicher & Schuell. Radionucleotides were purchased from Amersham. A  $\lambda$  Dash Fisher rat genomic library was obtained from Stratagene Cloning Systems.

**Isolation of a Rat D<sub>2A</sub> Gene.** A 15-kb phage recombinant clone encoding exons 2-8 was isolated as described (Todd et al., 1989). Fragments from this clone were used to screen a rat genomic library. Subsequent 5' walking clones were obtained with either a T<sub>3</sub> or a T<sub>7</sub> promoter to generate end-specific RNA probes as per the manufacturer's protocols. The exon 1 containing recombinant phage was isolated by hybridization with a 96-bp fragment encoding nucleotides 1-96 reported by Bunzow et al. (1988). Labeling of probes, hybridization, and washing conditions were performed in accordance with standard methods (Feinberg & Vogelstein, 1983; Maniatis et al., 1982).

**Oligonucleotides.** Oligonucleotide primers were synthesized by the Protein Chemistry Facility, Washington University, on an Applied Biosystems DNA synthesizer. Oligonucleotides used for this study were derived from the rat D<sub>2A</sub> receptor cDNA (Bunzow et al., 1988) and included the following:

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\* Address correspondence to this author.

<sup>†</sup> Department of Anatomy and Neurobiology.

<sup>§</sup> Departments of Psychiatry and Genetics.

<sup>1</sup> Abbreviations: bp, base pair(s); kb, kilobase; G-protein, guanine nucleotide binding protein; PCR, polymerase chain reaction.